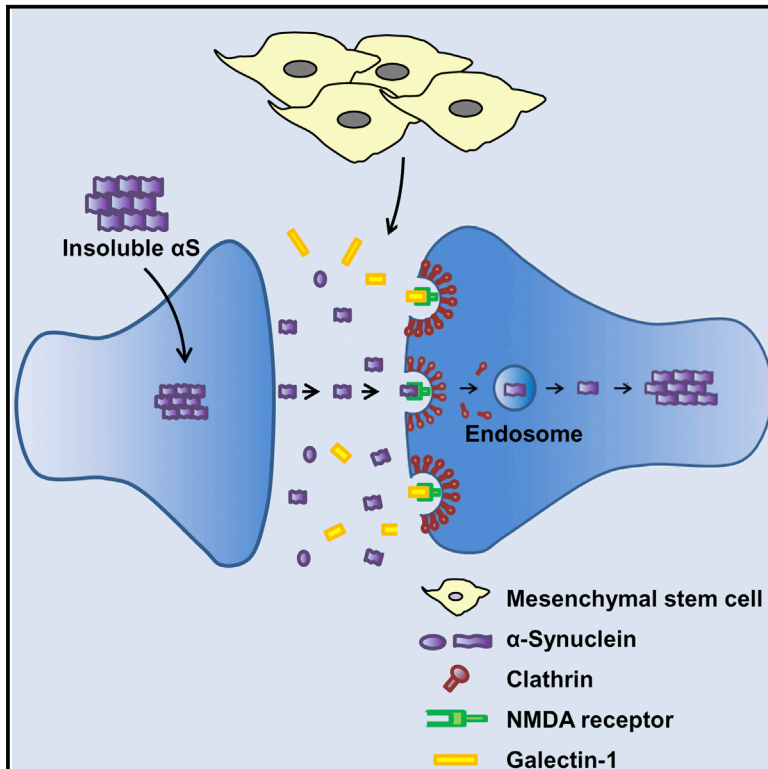


# Cell Reports

## Mesenchymal Stem Cells Inhibit Transmission of $\alpha$ -Synuclein by Modulating Clathrin-Mediated Endocytosis in a Parkinsonian Model

### Graphical Abstract



### Authors

Se Hee Oh, Ha Na Kim, Hyun Jung Park, ..., Mun Kyung Sunwoo, Seung-Jae Lee, Phil Hyu Lee

### Correspondence

phisland@chol.net

### In Brief

Using  $\alpha$ -synuclein-enriched models, Oh et al. show that MSCs exert neuroprotective properties through inhibition of cell-to-cell transmission of extracellular  $\alpha$ -synuclein.

### Highlights

- MSCs inhibit transmission of  $\alpha$ -synuclein by blocking the CME
- MSCs attenuate the induction of pathological  $\alpha$ -synuclein
- Galectin-1 is released by MSCs responsible for modulation of  $\alpha$ -synuclein
- MSCs lead to a prosurvival effect on neurons with improving motor function



# Mesenchymal Stem Cells Inhibit Transmission of $\alpha$ -Synuclein by Modulating Clathrin-Mediated Endocytosis in a Parkinsonian Model

Se Hee Oh,<sup>1,5</sup> Ha Na Kim,<sup>1,5</sup> Hyun Jung Park,<sup>1,2</sup> Jin Young Shin,<sup>1,2</sup> Eun-Jin Bae,<sup>4</sup> Mun Kyung Sunwoo,<sup>3</sup> Seung-Jae Lee,<sup>4</sup> and Phil Hyu Lee<sup>1,2,\*</sup>

<sup>1</sup>Department of Neurology, College of Medicine, Yonsei University, Seoul 120-752, Korea

<sup>2</sup>Severance Biomedical Science Institute, Yonsei University, Seoul 120-752, Korea

<sup>3</sup>Department of Neurology, Bundang Jesaeng General Hospital, Seongnam 463-774, Korea

<sup>4</sup>Department of Medicine, Neuroscience Research Institute, Seoul National University College of Medicine, Seoul 110-799, Korea

<sup>5</sup>Co-first author

\*Correspondence: [phisland@chol.net](mailto:phisland@chol.net)

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## SUMMARY

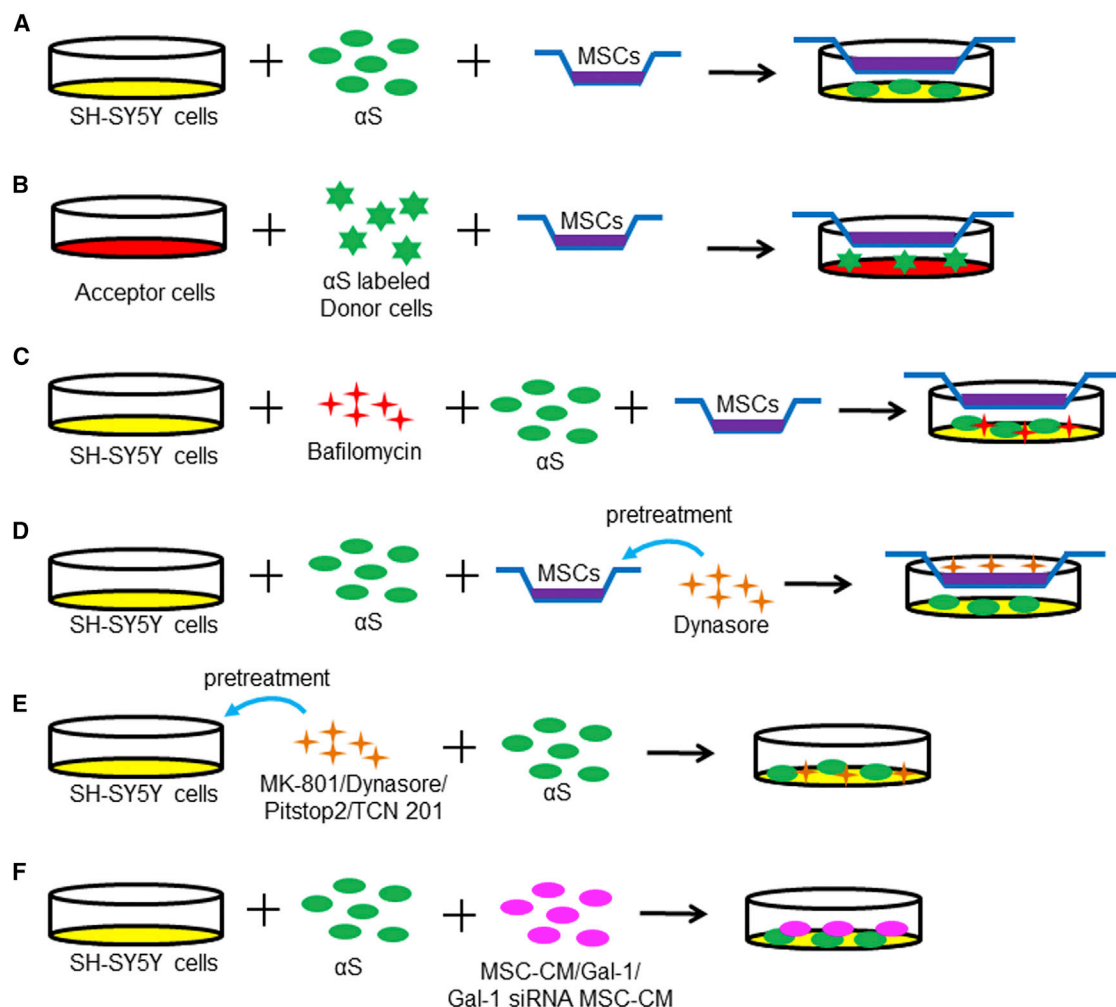
Ample evidence suggests that  $\alpha$ -synuclein is released from cells and propagated from one area of the brain to others via cell-to-cell transmission. In terms of their prion-like behavior,  $\alpha$ -synuclein propagation plays key roles in the pathogenesis and progression of  $\alpha$ -synucleinopathies. Using  $\alpha$ -synuclein-enriched models, we show that mesenchymal stem cells (MSCs) inhibited  $\alpha$ -synuclein transmission by blocking the clathrin-mediated endocytosis of extracellular  $\alpha$ -synuclein via modulation of the interaction with N-methyl-D-aspartate receptors, which led to a prosurvival effect on cortical and dopaminergic neurons with functional improvement of motor deficits in  $\alpha$ -synuclein-enriched models. Furthermore, we identify that galectin-1, a soluble factor derived from MSCs, played an important role in the transmission control of aggregated  $\alpha$ -synuclein in these models. The present data indicated that MSCs exert neuroprotective properties through inhibition of extracellular  $\alpha$ -synuclein transmission, suggesting that the property of MSCs may act as a disease-modifying therapy in subjects with  $\alpha$ -synucleinopathies.

## INTRODUCTION

$\alpha$ -Synuclein consists of 140 amino acids and is found naturally as an unfolded cytoplasmic protein in neuronal synaptic terminals. However, overexpression of  $\alpha$ -synuclein interrupts normal cell functions and leads to decreases in neurite outgrowth and cell adhesion (Takenouchi et al., 2001).  $\alpha$ -Synuclein aggregates comprising monomeric, oligomeric intermediate, or fibrillar forms are thought to be involved in a critical step in the pathogenesis of Parkinson's disease (PD) and in other  $\alpha$ -synucleinopathies, such as multiple system atrophy and dementia with Lewy bodies (Breydo et al., 2012; Ubhi et al., 2011; Uversky et al., 2001b).

Oligomeric and monomeric  $\alpha$ -synuclein have both been detected in cerebrospinal fluid and plasma samples from PD patients, suggesting that small aggregates of  $\alpha$ -synuclein access the extracellular space (Borghi et al., 2000; El-Agnaf et al., 2006; Lee et al., 2006). Previous animal and clinical data suggest that misfolded  $\alpha$ -synuclein can be released from cells by exocytosis and transmitted from one brain area to another via cell-to-cell propagation (Brundin et al., 2010; Hansen et al., 2011). Although the exact mechanism of  $\alpha$ -synuclein transmission remains unknown, evidence suggests that clathrin-mediated endocytosis (CME) may have an important role in internalization of extracellular  $\alpha$ -synuclein (Ben Gedalya et al., 2009; Cheng et al., 2011). As the cargo protein for endocytosis is usually recognized by a specific receptor on the cell surface (Carroll and Zukin, 2002; Lavezzari et al., 2004; van Dam and Stoorvogel, 2002), it is possible that  $\alpha$ -synuclein may interact with cell-surface receptors that have not been well specified until now. N-methyl-D-aspartate (NMDA) receptor subunits contain motifs that bind the endocytic adaptor protein involved in CME (Lavezzari et al., 2003). Additionally, a recent study provided the evidence that  $\alpha$ -synuclein could promote endocytic internalization of surface NMDA receptors through a mechanism requiring clathrin, suggesting an interaction between  $\alpha$ -synuclein and NMDA receptors (Cheng et al., 2011). Accordingly,  $\alpha$ -synuclein propagation from one area of the brain to others via cell-to-cell transmission is closely related with disease progression or clinical severity. Thus, strategies targeting modulation of  $\alpha$ -synuclein transmission may be important for the development of future disease-modifying therapies in individuals with  $\alpha$ -synucleinopathies.

Mesenchymal stem cells (MSCs) secrete various cytotrophic factors, including neurotrophic growth factors, chemokines, cytokines, and extracellular matrix protein, which, in turn, exert neuroprotective effects (Caplan and Dennis, 2006; Kim et al., 2012; Majumdar et al., 1998). In previous studies, we showed that MSCs have potent neuroprotective effects through modulation of neuroinflammation, inhibition of apoptotic cell death, increases in neurogenesis and neuronal differentiation, and enhancement of autophagy in neurodegenerative models (Kim et al., 2009; Park et al., 2011, 2012, 2014; Shin et al., 2014).



**Figure 1. Schematic Illustrations of In Vitro Experiments of  $\alpha$ -Synuclein Transmission**

(A) SH-SY5Y cells maintained on the bottom of a plate were co-cultured with MSCs using Costar transwell insert and simultaneously were treated with  $\alpha$ -synuclein ( $\alpha$ S) fibrils labeled with Alexa Fluor 488.

(B) A donor-acceptor co-culture method. The donor cells labeled with  $\alpha$ -synuclein were co-cultured on the top of the acceptor cells transfected with mCherry-tagged Rab5 (red). Simultaneously, these cells were co-cultured with MSCs using Costar transwell insert.

(C) Bafilomycin was simultaneously added to the SH-SY5Y and MSC co-cultures to exclude lysosomal degradation of  $\alpha$ -synuclein by SH-SY5Y.

(D) To exclude internalization of  $\alpha$ -synuclein by MSCs, MSCs were pre-treated with dynasore and then co-cultured with SH-SY5Y cells.

(E) The effects of treatment with MK-801, dynasore, Pitstop 2, or TCN 201 on internalization of  $\alpha$ -synuclein.

(F) The effects of MSC-CM, Gal-1 siRNA-treated MSC-CM, or Gal-1 treatment on internalization of  $\alpha$ -synuclein.

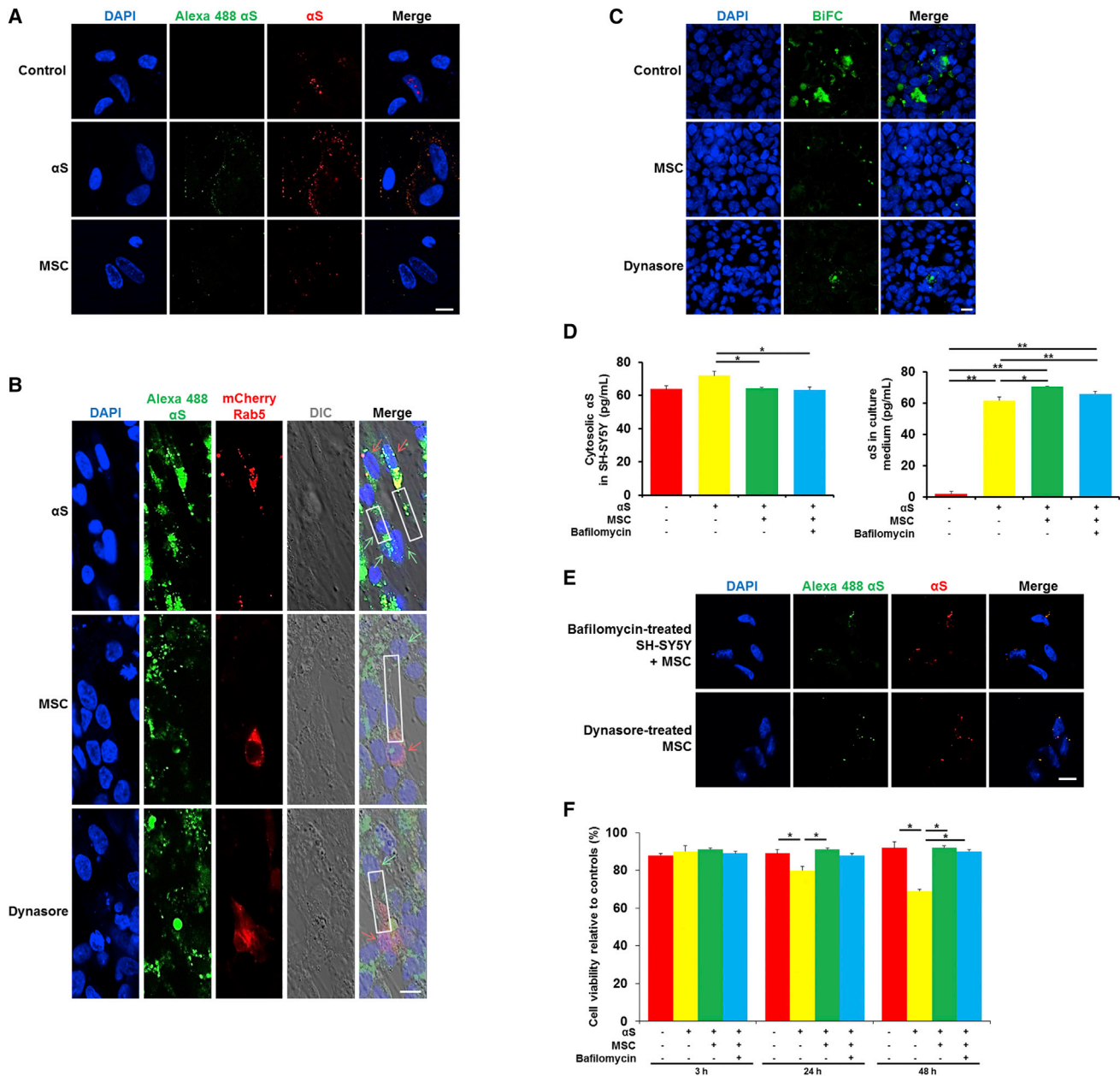
In the present study, we evaluated whether MSCs would inhibit transmission of extracellular  $\alpha$ -synuclein and thus exert a neuroprotective effect using  $\alpha$ -synuclein-enriched models. Furthermore, we determined that galectin-1 (Gal-1), the biological molecule secreted from MSCs, plays a crucial role in the modulation of extracellular  $\alpha$ -synuclein transmission.

## RESULTS

### MSCs Inhibit Internalization and Cell-to-Cell Transmission of Extracellular $\alpha$ -Synuclein in SH-SY5Y Cells

When  $\alpha$ -synuclein fibrils labeled with Alexa Fluor 488 were incubated in SH-SY5Y cells, punctate and vesicular patterns of

$\alpha$ -synuclein were revealed in the cytoplasm. Co-culture with MSCs (Figure 1A) markedly decreased internalization of labeled  $\alpha$ -synuclein fibrils (Figure 2A); however, co-culture with SH-SY5Y cells had no modulatory effects on  $\alpha$ -synuclein internalization (data not shown). A donor-acceptor co-culture method (Figure 1B) demonstrated that the transfer of  $\alpha$ -synuclein from donor cells to connected acceptor cells occurred in the  $\alpha$ -synuclein treatment group; however, transmission of  $\alpha$ -synuclein from donor cells to acceptor cells was observed infrequently in the group of co-cultured with MSCs or endocytosis blocker (dynasore) (Figure 2B). In order to demonstrate more clearly the transmission of  $\alpha$ -synuclein between cells, we used an assay based on biomolecular fluorescence complementation (BiFC), showing that BiFC signals were markedly decreased in the



**Figure 2. MSCs Inhibit Internalization and Cell-to-Cell Transmission of Extracellular  $\alpha$ -Synuclein**

(A) Immunostaining for internalization of labeled  $\alpha$ -synuclein ( $\alpha$ S, green) in SH-SY5Y cells after treatment with  $\alpha$ S or co-culture with MSCs (MSC). Scale bar represents 10  $\mu$ m.

(B) A donor-acceptor co-culture method for transmission (white boxed area) of  $\alpha$ S from donor cells (green arrows) to connected acceptor cells (red arrows) after either  $\alpha$ S treatment alone, co-culture with MSCs, or dynasore. Scale bar represents 10  $\mu$ m. DIC, differential interference contrast.

(C) BiFC signals (green) in the MSC or dynasore treatment group compared with the control group. Scale bar represents 20  $\mu$ m.

(D) Quantification of internalized cytosolic  $\alpha$ S and extracellular  $\alpha$ S in the culture medium of the  $\alpha$ S treatment group, the co-culture group with MSCs, and the bafilomycin-treated MSC group ( $n = 3$  per group).

(E) Immunostaining for internalization of labeled  $\alpha$ S in SH-SY5Y cells co-cultured with MSCs after treatment with either bafilomycin or dynasore. Scale bar represents 10  $\mu$ m.

(F) Quantification of cell viability in the  $\alpha$ S group, the co-culture group with MSCs, and the bafilomycin-treated MSC group for 3 hr, 24 hr, and 48 hr ( $n = 3$  per group).

All data are presented as means  $\pm$  SE. \* $p < 0.05$ ; \*\* $p < 0.01$ .



MSC or dynasore treatment group compared with the control group (Figure 2C). Thus, co-culture with MSCs significantly decreased the levels of internalized cytosolic  $\alpha$ -synuclein, with a concomitant increase in extracellular  $\alpha$ -synuclein of culture medium, compared with those of the  $\alpha$ -synuclein treatment group (Figure 2D). To exclude lysosomal degradation of  $\alpha$ -synuclein by SH-SY5Y and internalization of  $\alpha$ -synuclein by MSCs, a lysosomal inhibitor (bafilomycin) and dynasore were added to the SH-SY5Y and MSC co-cultures, respectively (Figures 1C and 1D). These drugs did not influence  $\alpha$ -synuclein internalization or change the intracellular and extracellular  $\alpha$ -synuclein levels (Figures 2D and 2E), indicating that soluble factors secreted from MSCs might be responsible for modulation of  $\alpha$ -synuclein internalization. To exclude the mitogenic proliferation effects of MSCs, we additionally performed a viability test using differentiated SH-SY5Y cells that exhibit no immunoreactivity with Ki-67 antibody. Incubation of  $\alpha$ -synuclein fibrils for 24 hr and 48 hr decreased SH-SY5Y cell viability, whereas co-culture with MSCs recovered  $\alpha$ -synuclein-induced cell death (Figure 2F).

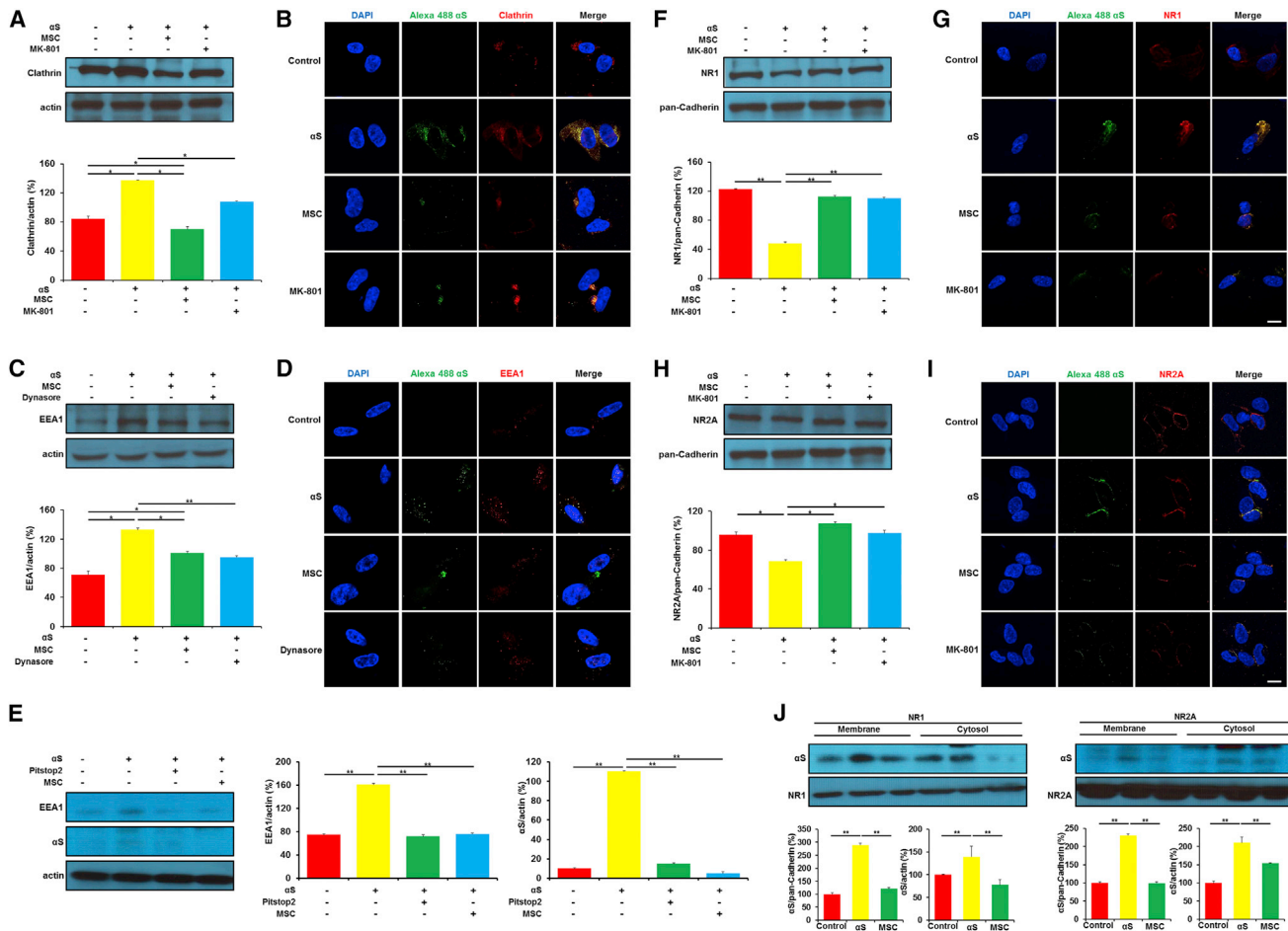
### MSCs Inhibit CME of Extracellular $\alpha$ -Synuclein Fibrils through Modulation of Surface NMDA Receptors

We examined the expression and immunoreactivity of clathrin to determine the role of CME in  $\alpha$ -synuclein internalization. When  $\alpha$ -synuclein fibrils were incubated in SH-SY5Y cells, the expression of clathrin was significantly increased, compared with that in the control. However, co-culture with MSCs or treatment with MK-801 (Figures 1A and 1E), a noncompetitive NMDA receptor antagonist, significantly attenuated the expression of  $\alpha$ -synuclein-induced clathrin (Figure 3A). Using confocal microscopy, we confirmed that co-culture with MSCs or treatment with MK-801 in  $\alpha$ -synuclein-treated SH-SY5Y cells markedly decreased the immunoreactivity of clathrin that was co-localized with  $\alpha$ -synuclein (Figure 3B). Additionally, the expression of early endosome antigen 1 (EEA1) was increased significantly in  $\alpha$ -synuclein-treated SH-SY5Y cells compared with that in the control. However, co-culture with MSCs or treatment with dynasore (Figures 1A and 1E) significantly decreased the expression of EEA1, the level of which corresponded to that of an endocytosis inhibitor treatment (Figure 3C). Confocal microscopy showed that co-culture with MSCs or dynasore treatment in  $\alpha$ -synuclein-treated SH-SY5Y cells markedly decreased the immunoreactivity of EEA1 that was co-localized with  $\alpha$ -synuclein (Figure 3D). When clathrin-inhibitor (Pitstop 2)-treated SH-SY5Y cells were incubated with  $\alpha$ -synuclein (Figure 1E), the expression of intracellular  $\alpha$ -synuclein and EEA1 was significantly decreased compared to that in only  $\alpha$ -synuclein-treated SH-SY5Y cells (Figure 3E). Next, we identified whether MSCs inhibit the interaction between  $\alpha$ -synuclein and NMDA receptors using extraction of cell-surface membrane.  $\alpha$ -Synuclein treatment in SH-SY5Y cells led to a significant attenuation in the expression of surface NR1 and NR2A subunits relative to control. However, co-culture with MSCs or MK-801 treatment (Figures 1A and 1E) prevented a decrease in the expression of  $\alpha$ -synuclein-induced NR1 and NR2A subunits (Figures 3F and 3H). Moreover, immunocytochemical analysis showed that  $\alpha$ -synuclein treatment increased the immunoreactivity of surface NR1 and NR2A subunits that

were co-localized with  $\alpha$ -synuclein, whereas MSCs or MK-801 treatment led to a decrease in immunoreactivity of co-merged  $\alpha$ -synuclein and surface NR1 and NR2A subunits (Figures 3G and 3I). Additionally, we found that a selective NR1 and NR2A receptor antagonist (TCN 201) treatment in  $\alpha$ -synuclein-treated SH-SY5Y cells (Figure 1E) significantly increased the expression of  $\alpha$ -synuclein-induced NR1 and NR2A subunits and attenuated the expression of  $\alpha$ -synuclein-induced clathrin (Figure S1A). An immunoprecipitation assay to clarify the interaction of  $\alpha$ -synuclein and NMDA receptors showed that  $\alpha$ -synuclein treatment increased the amount of NR1 or NR2A immunoprecipitated with  $\alpha$ -synuclein, whereas the amount of NR1 or NR2A that interacted with  $\alpha$ -synuclein in the MSC-treated group was comparable to that of the control group (Figure 3J). As expected, treatment with dynasore or MK801 alone in SH-SY5Y cells had no effect on immunoreactivities or protein expressions of clathrin, EEA1, NR1, and NR2A (Figures S1B and S1C).

### Identification and Characterization of MSC-Derived Factors

To determine proteins secreted from MSCs, we collected three independent samples of fresh medium, SH-SY5Y-conditioned media (CM), and MSC-CM for two-dimensional (2D)-PAGE and MALDI-TOF mass spectrometry (MALDI-TOF/MS) proteomics (Figures 4A and 4B). Of the proteins expressed exclusively in MSC-CM (Table S1), we selected Gal-1 as a candidate for  $\alpha$ -synuclein modulation. We identified that Gal-1 was expressed within MSCs injected intravenously in  $\alpha$ -synuclein-inoculated animals by showing that human-specific nuclear-mitotic-apparatus-protein (NuMA)-positive cells were co-immunostained with Gal-1 (Figure S2A) and that the expression of this protein was significantly increased in MSCs-treated animals (Figure S2B). Next, we evaluated whether Gal-1 can modulate transmission of  $\alpha$ -synuclein (Figure 1F). Gal-1 treatment in  $\alpha$ -synuclein-treated SH-SY5Y cells led to decreased internalization of labeled  $\alpha$ -synuclein (Figure 4C), with a concomitant decrease in expressions of clathrin and EEA1 as well as increased expression of NR1 (Figure 4D), which was followed by increased neuronal viability (Figure 4E). When MSCs were treated with Gal-1 small interfering RNA (siRNA) (Figure S2C), Gal-1 siRNA counteracted the inhibitory effect of MSCs on CME of  $\alpha$ -synuclein via NR1 subunit (Figures 4C and 4D). Moreover, SH-SY5Y cell viability was prominently attenuated in the presence of Gal-1 siRNA-treated MSC-CM (Figure 4E). However, the intensity of  $\alpha$ -synuclein aggregates, transmission, and neuronal viability in Gal-1 siRNA-treated MSC-CM was not comparable to the corresponding values in fresh medium or SH-SY5Y-CM (Figures 4C–4E), suggesting that other soluble factors may be involved in extracellular  $\alpha$ -synuclein modulation. To investigate the interaction between Gal-1 and NR1 and NR2A receptors, the membrane and cytosol lysates were immunoprecipitated with anti-NR1 or anti-NR2A and then immunoblotted with an antibody against Gal-1. We found that MSC-CM treatment significantly increased the amount of Gal-1 immunoprecipitated with NR1 in both membrane and cytosol fractions, whereas the amount of Gal-1 that interacted with NR1 and NR2A in the fresh medium group was comparable to that of the control group (Figure 4F).



**Figure 3. MSCs Inhibit CME of Extracellular  $\alpha$ -Synuclein Fibrils via Modulation of Surface NMDA Receptors**

(A and B) Western blot for clathrin (A) and immunostaining of clathrin that is co-localized with  $\alpha$ -synuclein ( $\alpha$ S) (B) after  $\alpha$ S treatment alone, co-culture with MSCs, or MK-801 treatment (n = 3 per group). Scale bar represents 10  $\mu$ m.

(C and D) Western blot for EEA1 (C) and immunostaining of EEA1 that is co-localized with  $\alpha$ S (D) after either  $\alpha$ S treatment alone, co-culture with MSCs, or dynasore treatment (n = 3 per group). Scale bar represents 10  $\mu$ m.

(E) Western blot for EEA1 and  $\alpha$ S after  $\alpha$ S treatment alone, Pitstop 2 treatment, or co-culture with MSC (n = 3 per group).

(F and G) Western blot for NR1 (F) and immunostaining of NR1 that is co-localized with  $\alpha$ S (G) after either  $\alpha$ S treatment alone, co-culture with MSCs, or MK-801 treatment (n = 3 per group). Scale bar represents 10  $\mu$ m.

(H and I) Western blot for NR2A (H) and immunostaining of NR2A that is co-localized with  $\alpha$ S (I) after either  $\alpha$ S treatment alone, co-culture with MSCs, or MK-801 treatment (n = 3 per group). Scale bar represents 10  $\mu$ m.

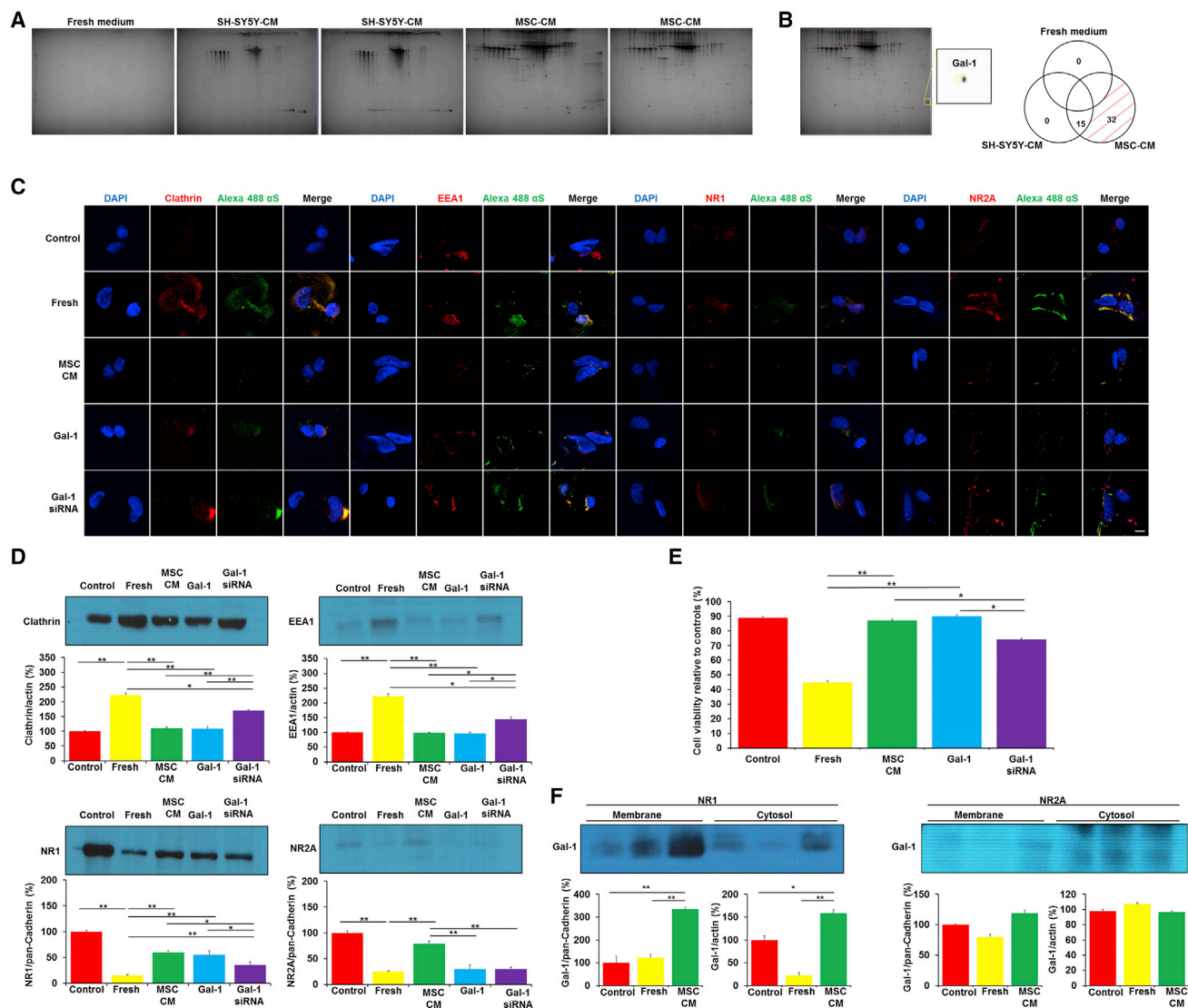
(J) The membrane and cytosol lysates that were immunoprecipitated with anti-NR1 or NR2A and then immunoblotted with anti- $\alpha$ S. The blots reprobed with anti-NR1 or NR2A antibody to illustrate that relatively equivalent amounts of NMDA receptor were expressed among the groups (n = 3 per group).

All data are presented as means  $\pm$  SE. \*p < 0.05; \*\*p < 0.01.

Meanwhile, there was no direct interaction between Gal-1 and  $\alpha$ -synuclein (Figure S3A). Additionally, we compared the patterns of cytokines between MSC-CM and Gal-1 siRNA-treated MSC-CM in  $\alpha$ -synuclein-treated SH-SY5Y cells to evaluate whether siRNA Gal-1 of MSC abrogates the immunomodulatory effects. The pro-inflammatory cytokine levels of interleukin (IL)-6, chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), and interferon (IFN)- $\gamma$  and the anti-inflammatory cytokine levels of IL-4, IL-10, hepatocyte growth factor (HGF), and transforming growth factor (TGF)- $\beta$ 1 did not differ between the MSC-CM and siRNA Gal-1-treated MSC-CM groups (Figure S3B).

### Short-Term Effects of MSCs on Transmission of Extracellular $\alpha$ -Synuclein in $\alpha$ -Synuclein-Inoculated Animals

Following stereotaxic inoculation of Alexa Fluor 488-labeled  $\alpha$ -synuclein fibrils into the cortex of mice (Figures S4A and S4C), we examined internalized  $\alpha$ -synuclein that was detected as fluorescent punctate within the cytoplasm at 7 days after  $\alpha$ -synuclein inoculation. The immunoreactivity of  $\alpha$ -synuclein was more densely observed in the cortical areas neighboring the inoculation site and extensively detected from the ipsilateral cortex to the contralateral hemisphere (Figure 5A). However, MSC treatment markedly decreased the density of internalized



**Figure 4. Gal-1 Plays an Important Role in Transmission Control of Extracellular  $\alpha$ -Synuclein**

(A and B) 2D-PAGE and MALDI-TOF/MS proteomics approaches from MSC-CM. On the 2D-PAGE gel of total protein extracted from each medium at 72 hr, spot intensities and patterns were largely similar between independent samples from the SH-SY5Y-CM group and MSC-CM group. (A) Fresh medium contained 6 discernable polypeptides, SH-SY5Y-CM contained 87 and 96 discernable polypeptides, and MSC-CM contained 189 and 157 discernable polypeptides by silver staining. (B) The analysis identified 47 spots in MSC-CM that differed significantly by at least 2-fold in expression level compared with fresh or SH-SY5Y-CM. The area shown in the yellow box in the left panel is enlarged in the right panels. Gal-1 protein is indicated by a yellow circle in enlarged image. A Venn diagram showed that MSC-CM contained 32 spots, including Gal-1, which were not shared by fresh medium and SH-SY5Y-CM.

(C) Immunostaining for clathrin, EEA1, and surface NR1 and NR2A in SH-SY5Y cells that are co-localized with  $\alpha$ -synuclein ( $\alpha$ S) in the presence of fresh medium, MSC-CM, Gal-1 treatment, or Gal-1 siRNA-treated MSC-CM. Scale bar represents 10  $\mu$ m.

(D) Western blot for clathrin, EEA1, and surface NR1 and NR2A in SH-SY5Y cells after treatment with either Gal-1 or Gal-1 siRNA-treated MSC-CM compared with fresh medium or MSC-CM ( $n = 3$ , each group).

(E) A viability assay in SH-SY5Y cells after  $\alpha$ S fibrils were treated with either Gal-1 or Gal-1 siRNA-treated MSC-CM compared with fresh medium or MSC-CM ( $n = 5$ , each group).

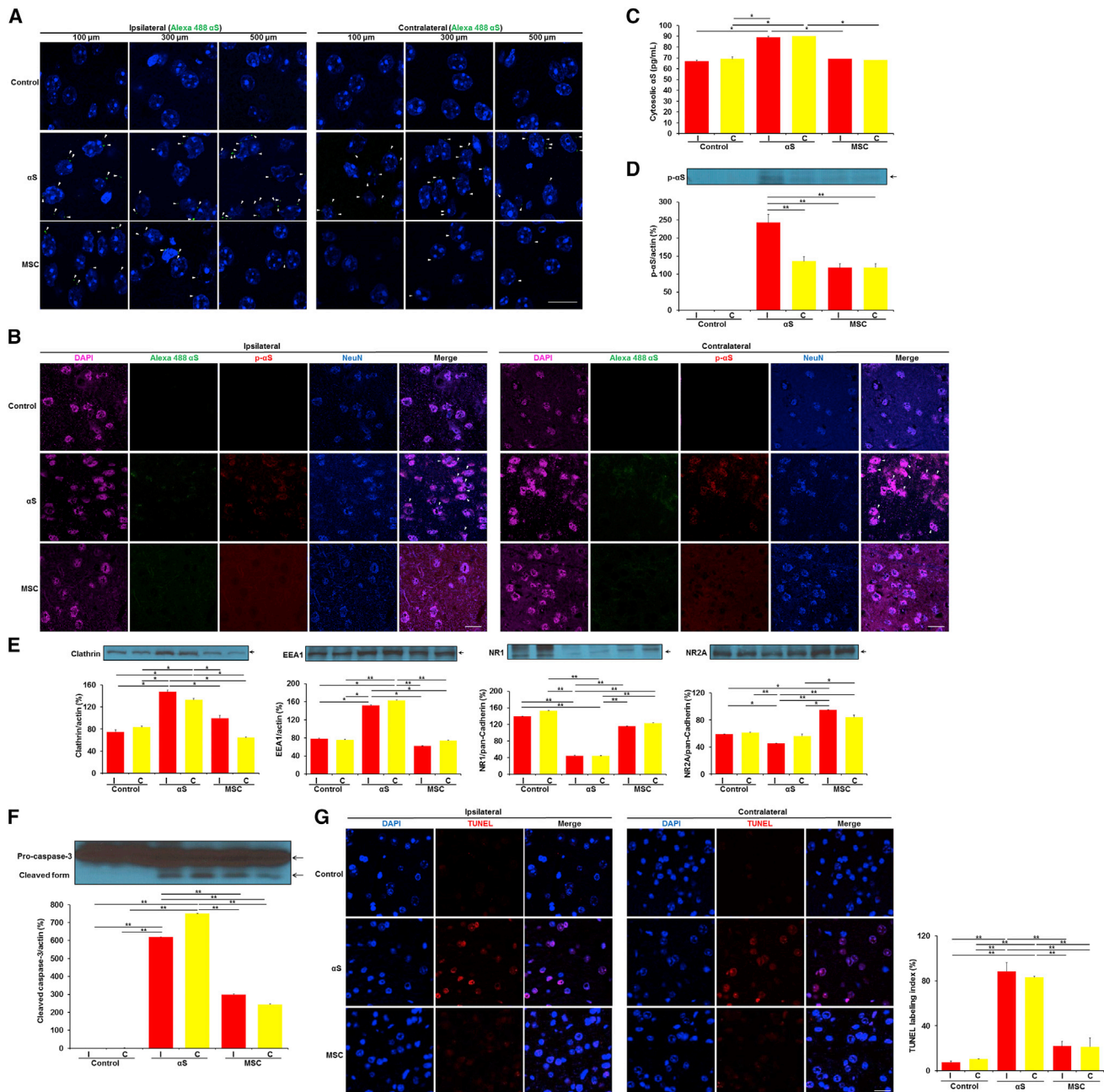
(F) The membrane and cytosol lysates that were immunoprecipitated with anti-NR1 or NR2A and then immunoblotted with anti-Gal-1 ( $n = 3$  per group).

All data are presented as means  $\pm$  SE. \* $p < 0.05$ ; \*\* $p < 0.01$ .

$\alpha$ -synuclein in both ipsilateral and contralateral hemispheres (Figure 5A). Moreover, we examined the immunoreactivity of phosphorylated  $\alpha$ -synuclein to evaluate whether exogenous  $\alpha$ -synuclein induces pathogenic  $\alpha$ -synuclein. The phosphorylated  $\alpha$ -synuclein was exclusively immunostained in the ipsilat-

eral and contralateral hemispheres of  $\alpha$ -synuclein-inoculated animals, and this immunoreactivity was observed within neurons in the cortex (Figure 5B). However, phosphorylated  $\alpha$ -synuclein immunoreactivity was not observed in MSC-treated  $\alpha$ -synuclein-inoculated animals (Figure 5B). The ELISA analysis showed





**Figure 5. Short-Term Effects of MSCs on Extracellular  $\alpha$ -Synuclein Transmission**

(A) Immunostaining for internalized Alexa Fluor 488-labeled  $\alpha$ -synuclein ( $\alpha$ S), showing that the density and extent of spreading  $\alpha$ S in ipsilateral and contralateral hemispheres were less prominent in mice receiving MSCs compared with  $\alpha$ S alone. Scale bar represents 10  $\mu$ m. Arrowheads denote labeled  $\alpha$ S in the cortical areas.

(B) The phosphorylated  $\alpha$ -synuclein (p- $\alpha$ S) was immunostained within neurons of  $\alpha$ S-inoculated ipsilateral and contralateral hemispheres, whereas this immunoreactivity was not observed in MSC-treated animals. Scale bar represents 10  $\mu$ m. Arrowheads denote co-localization of Alexa Fluor 488-labeled  $\alpha$ S, p- $\alpha$ S, and neuronal nuclei (NeuN).

(C) Quantification of cytosolic  $\alpha$ S using ELISA in ipsilateral and contralateral hemispheres of mice receiving MSCs compared with  $\alpha$ S alone (n = 5, per group).

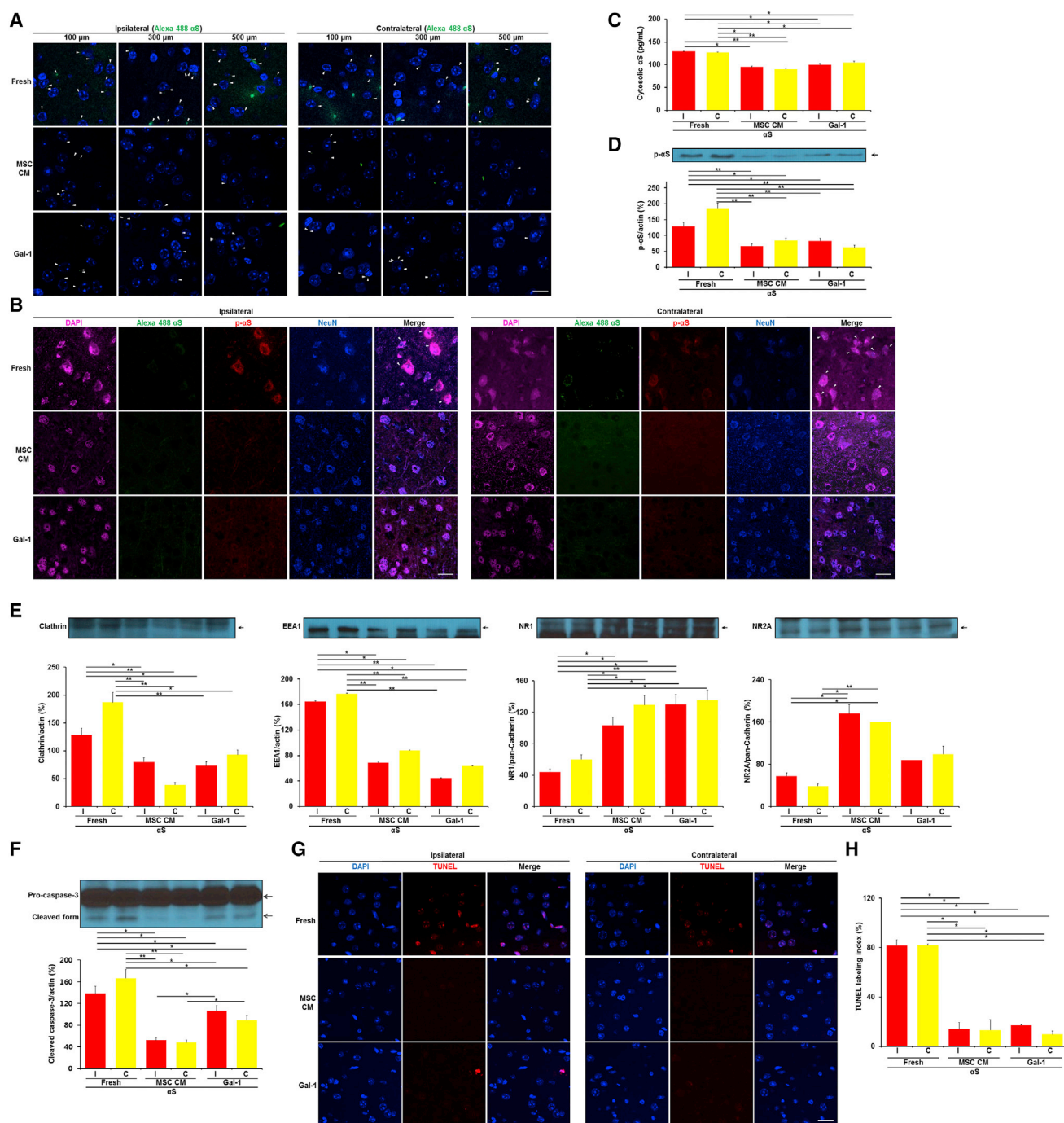
(D) Western blot for p- $\alpha$ S in ipsilateral and contralateral hemispheres of mice receiving MSCs compared with  $\alpha$ S alone (n = 5 per group).

(E) Western blot for clathrin, EEA1, NR1 subunit, and NR2A subunit in ipsilateral (I) and contralateral (C) hemispheres of mice receiving MSCs compared with  $\alpha$ S alone (n = 5 per group).

(F and G) Western blot for pro- and cleaved caspase-3 (F) and immunostaining of TUNEL (G) in mice after treatment with MSCs compared with  $\alpha$ S alone. Scale bar represents 10  $\mu$ m.

All data are presented as means  $\pm$  SE. \*p < 0.05; \*\*p < 0.01.





**Figure 6. Treatment with Either MSC-CM or Gal-1 Modulates CME of Extracellular  $\alpha$ -Synuclein Fibrils and Reduces the Cell-to-Cell Transmission**

(A) Immunostaining for internalized Alexa Fluor 488-labeled  $\alpha$ -synuclein ( $\alpha$ S), showing that the density and extent of propagated  $\alpha$ S in ipsilateral (I) and contralateral (C) hemispheres were less prominent in mice receiving MSC-CM or Gal-1. Scale bar represents 10  $\mu$ m. Arrowheads denote Alexa Fluor 488-labeled  $\alpha$ S in the cortical areas neighboring the inoculation site.

(B) Immunostaining of Alexa Fluor 488-labeled  $\alpha$ S and phosphorylated  $\alpha$ -synuclein (p- $\alpha$ S) within neurons of ipsilateral and contralateral hemispheres were less prominent in treatment with either MSC-CM or Gal-1 compared with fresh medium (Fresh). Scale bar represents 10  $\mu$ m. Arrowheads denote co-localization of Alexa Fluor 488-labeled  $\alpha$ S, p- $\alpha$ S, and NeuN.

(C) Quantification of cytosolic  $\alpha$ S using ELISA in ipsilateral and contralateral hemispheres of mice receiving MSC-CM or Gal-1 (n = 5 per group).

(D) Western blot for p- $\alpha$ S in ipsilateral and contralateral hemispheres of mice receiving MSC-CM or Gal-1 (n = 5 per group).

(legend continued on next page)

that MSC treatment in  $\alpha$ -synuclein-treated mice significantly attenuated the expression of cytosolic  $\alpha$ -synuclein in both the ipsilateral and contralateral hemispheres compared with that in mice treated with  $\alpha$ -synuclein alone (Figure 5C). In addition, MSC treatment in  $\alpha$ -synuclein-inoculated brain significantly attenuated phosphorylated  $\alpha$ -synuclein expression (Figure 5D). Next, we examined whether MSC administration modulates CME of extracellular  $\alpha$ -synuclein fibrils through the modulation of surface NMDA receptors. An inoculation of  $\alpha$ -synuclein fibrils led to increased expression of clathrin in both the ipsilateral and contralateral hemispheres of inoculation, whereas MSC treatment significantly attenuated expression of this protein (Figure 5E). Immunohistochemical analysis showed that MSC treatment led to a decrease in the immunoreactivity of labeled  $\alpha$ -synuclein and clathrin in both the ipsilateral and contralateral hemispheres (Figure S5). As a result of CME inhibition, MSC administration in  $\alpha$ -synuclein-inoculated mice significantly decreased expression of EEA1 (Figure 5E) and the immunoreactivity of labeled  $\alpha$ -synuclein and EEA1 in both the ipsilateral and contralateral hemispheres (Figure S5). Additionally, MSC administration led to a significant increase in the expression of  $\alpha$ -synuclein-induced surface NR1 and NR2A subunits (Figure 5E) and a decrease in the immunoreactivity of surface NR1 and NR2A subunits that were co-localized with  $\alpha$ -synuclein (Figure S5) in both the ipsilateral and contralateral hemispheres of  $\alpha$ -synuclein-inoculated mice. Consequently,  $\alpha$ -synuclein inoculation led to markedly increased expression of cleaved caspase-3 and the immunoreactivity of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) in the ipsilateral hemisphere relative to controls; however, MSC treatment notably decreased the expression of cleaved caspase-3 and the number of TUNEL-positive neurons (Figures 5F and 5G). To exclude microglial effects on  $\alpha$ -synuclein modulation via microglial clearance of  $\alpha$ -synuclein fibrils, we further analyzed the short-term effects of MSC on  $\alpha$ -synuclein propagation in  $\alpha$ -synuclein-inoculated animals by using both M1 and M2 microglia depletion conditions. MSC treatment in microglia depletion conditions markedly decreased the immunoreactivities of internalized  $\alpha$ -synuclein and phosphorylated  $\alpha$ -synuclein in both the ipsilateral and contralateral hemispheres with a significant decrease in the expression of cytosolic  $\alpha$ -synuclein and phosphorylated  $\alpha$ -synuclein (Figure S6). This indicates that the effect of MSC on  $\alpha$ -synuclein propagation may not be mediated by immunomodulation but rather the consequence of a direct inhibition of  $\alpha$ -synuclein transmission.

### Transmission of Extracellular $\alpha$ -Synuclein Is Decreased by Gal-1 in $\alpha$ -Synuclein-Inoculated Animals

We evaluated whether Gal-1 can modulate  $\alpha$ -synuclein transmission in  $\alpha$ -synuclein-inoculated animals. Inhibition of  $\alpha$ -synuclein propagation was similarly observed following MSC-CM or Gal-1 treatment (Figure 6A). Additionally, MSC-CM or Gal-1

treatment markedly decreased the immunoreactivity of phosphorylated  $\alpha$ -synuclein in the ipsilateral and contralateral hemispheres of  $\alpha$ -synuclein-inoculated animals (Figure 6B). The ELISA analysis confirmed that the expression of cytosolic and phosphorylated  $\alpha$ -synuclein was significantly decreased in the ipsilateral and contralateral hemispheres following MSC-CM or Gal-1 (Figures 6C and 6D). MSC-CM or Gal-1 treatment significantly attenuated clathrin expression (Figure 6E) and immunoreactivity (Figure S5) in both the ipsilateral and contralateral hemispheres of inoculation, which was accompanied by decreased expression of EEA1 (Figure 6E) and the immunoreactivity of labeled  $\alpha$ -synuclein and EEA1 (Figure S5). MSC-CM or Gal-1 treatment inhibited the CME of extracellular  $\alpha$ -synuclein fibrils through modulation of surface NMDA receptors; however, Gal-1 treatment did not show a significant interaction with the surface NR2A subunit (Figure 6E). In addition, we evaluated whether siRNA Gal-1 of MSC could influence microglia polarization to exclude microglial clearance of  $\alpha$ -synuclein as much as possible. The levels of IL-1 $\beta$  (an M1 marker) and arginase 1 (an M2 marker) did not differ significantly between the MSC-CM and siRNA Gal-1-treated MSC-CM groups (Figure S7A). Furthermore, we showed that CD105 siRNA-treated MSC-CM did not change the immunoreactivity and expression patterns of phosphorylated  $\alpha$ -synuclein compared to the MSC-CM treatment, indicating that CD105 released by MSCs does not influence  $\alpha$ -synuclein phosphorylation (Figures S7B and S7C). Consequently, MSC or Gal-1 treatment notably decreased the expression of cleaved caspase-3 and the number of TUNEL-positive neurons in  $\alpha$ -synuclein-inoculated animals (Figures 6F–6H).

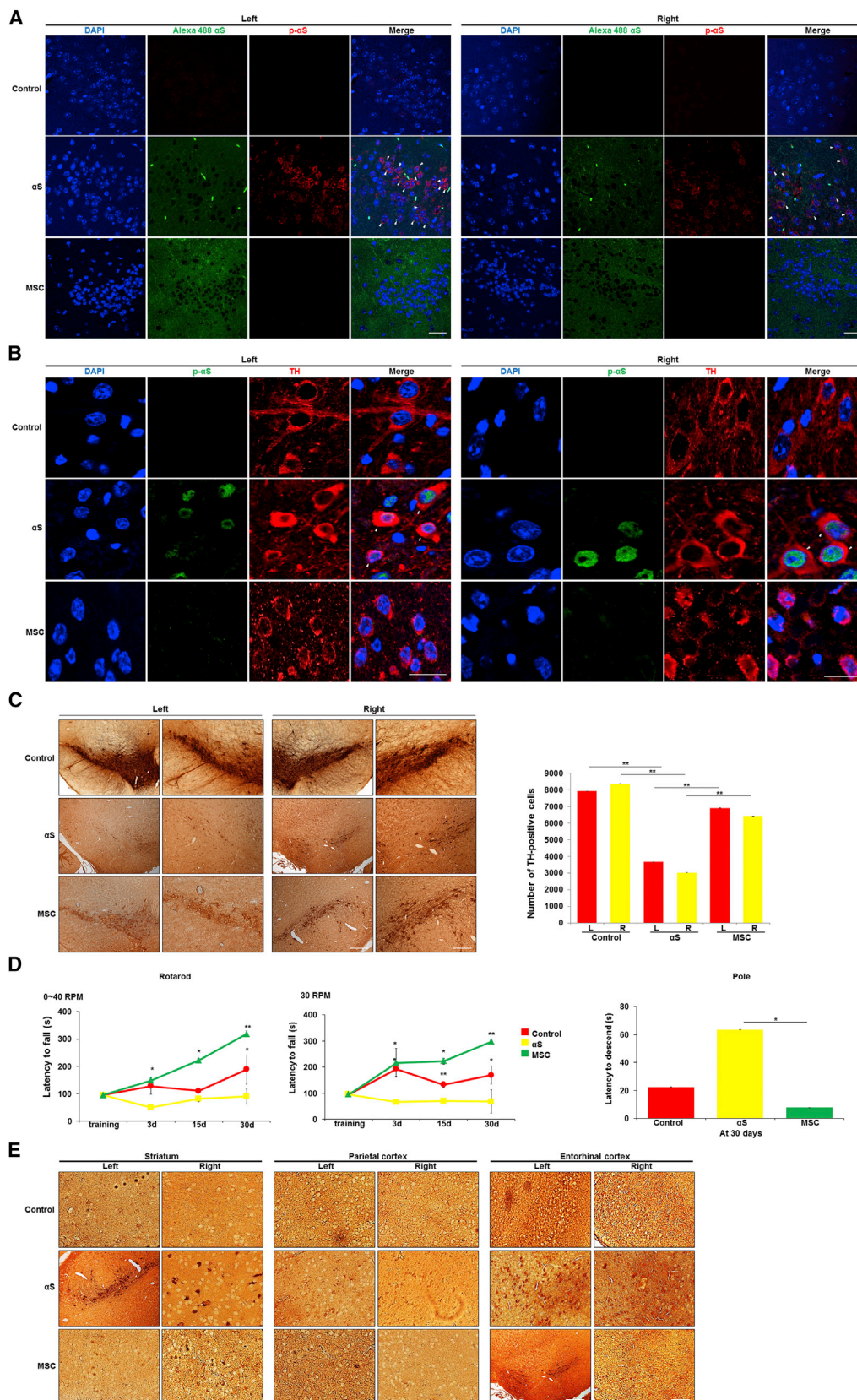
### Long-Term Effects of MSCs on Transmission of Extracellular $\alpha$ -Synuclein in $\alpha$ -Synuclein-Inoculated Animals

Following stereotaxic inoculation of Alexa Fluor 488-labeled  $\alpha$ -synuclein fibrils into the dorsal striatum of mice (Figures S4B and S4D), we assessed dopaminergic neuronal loss in the substantia nigra (SN) of the midbrain and behavioral deficits at 30 days after  $\alpha$ -synuclein inoculation.  $\alpha$ -Synuclein inoculation in the striatum markedly increased the immunoreactivity of the phosphorylated form of  $\alpha$ -synuclein in the midbrain (Figure 7A) and dopaminergic neurons (Figure 7B), which was accompanied by a significant decrease in the number of tyrosine hydroxylase (TH)-positive neurons in the SN (Figure 7C). On behavioral analysis,  $\alpha$ -synuclein inoculation led to progressive patterns of the latency to fall on the Rotarod test and increased latency to descend on the latency to fall test at 30 days after  $\alpha$ -synuclein inoculation compared to the control group (Figure 7D). However, MSC treatment in  $\alpha$ -synuclein-inoculated animals significantly attenuated the immunoreactivity of the phosphorylated form of  $\alpha$ -synuclein in the midbrain (Figure 7A) and dopaminergic neurons (Figure 7B). Moreover, MSC treatment significantly decreased dopaminergic neuronal loss in the SN (Figure 7C)

(E) Western blot for clathrin, EEA1, NR1 subunit, and NR2A subunit in ipsilateral and contralateral hemispheres of mice receiving MSC-CM or Gal-1 ( $n = 5$  per group).

(F–H) Western blot for pro- and cleaved caspase-3 (F) and immunostaining of TUNEL (G and H) in mice after treatment with MSC-CM or Gal-1. Scale bar represents 10  $\mu$ m.

All data are presented as means  $\pm$  SE. \* $p < 0.05$ ; \*\* $p < 0.01$ .



(legend on next page)



and led to the restoration of impaired motor coordination and balance on the Rotarod test and pole test (Figure 7D). In addition, phosphorylated  $\alpha$ -synuclein was observed in the striatal areas neighboring the inoculation site and was extensively detected in the bilateral parietal and entorhinal cortices at 30 days after  $\alpha$ -synuclein inoculation (Figure 7E). However, MSC treatment significantly attenuated the immunoreactivity of phosphorylated  $\alpha$ -synuclein in these areas (Figure 7E).

## DISCUSSION

The present study demonstrated that MSCs have inhibited propagation of  $\alpha$ -synuclein via modulation of its transmission by inhibiting NMDA-receptor-mediated endocytosis, which led to a prosurvival effect on neurons with functional improvement of motor deficits in  $\alpha$ -synuclein-enriched models. In addition, we found that Gal-1, soluble factor derived from MSCs, plays an important role in transmission control of extracellular  $\alpha$ -synuclein via competing with  $\alpha$ -synuclein for NMDA receptor binding in these models. Our data suggest that the property of MSCs in modulating the propagation of extracellular  $\alpha$ -synuclein may be applicable to future clinical strategies for treatment of patients with  $\alpha$ -synucleinopathies.

$\alpha$ -Synuclein has a tendency to aggregate and accumulate, thus forming small intracellular aggregates, which could lead to an increase in cellular toxicity and cell death in various types of  $\alpha$ -synucleinopathies (Conway et al., 1998; Uversky et al., 2001a). Importantly, recent studies have provided evidence for cell-to-cell propagation of  $\alpha$ -synuclein (Lee et al., 2010b, 2014), showing that  $\alpha$ -synuclein and its aggregates are released from neuronal cells via exocytosis (Lee et al., 2005, 2014; Li et al., 2008) and that neurons and glial cells have the ability to internalize extracellular  $\alpha$ -synuclein aggregates through endocytosis (Desplats et al., 2009; Guo and Lee, 2014; Lee et al., 2010a). In terms of their prion-like behavior, extracellular  $\alpha$ -synuclein aggregates seem to play key roles in the pathogenesis and progression of  $\alpha$ -synucleinopathies; therefore, treatment strategies that are focused on the modulation of extracellular  $\alpha$ -synuclein transmission would be clinically relevant. In this regard, the results of immunotherapy in animal models of Lewy body diseases are very suggestive, because antibodies against  $\alpha$ -synuclein can modulate aggregated  $\alpha$ -synuclein at several steps of accumulation and propagation (Bae et al., 2012; Fagerqvist et al., 2013; Masliah et al., 2011; Tran et al., 2014; Valera and Masliah, 2013). Therefore, a therapeutic strategy to inhibit propagation

of extracellular  $\alpha$ -synuclein aggregates may be an important pharmacological target in disease-modifying treatment strategies for  $\alpha$ -synucleinopathies.

Several studies have demonstrated that MSCs exert neuroprotective effects by secretion of neurotropic molecules that directly or indirectly can modulate neurodegenerative microenvironment (Mathieu et al., 2012; Paul and Anisimov, 2013). Importantly, even though the underlying mechanisms of MSCs seem to differ depending on disease entity or animal models of parkinsonian diseases, MSCs could exert a neuroprotective effect on dopaminergic neurons by autophagic modulation of  $\alpha$ -synuclein in the mitochondrial-neurotoxin-induced PD model and immunomodulation (Park et al., 2014; Stemberger et al., 2011). Here, we found that MSCs have the ability to inhibit  $\alpha$ -synuclein endocytosis and lead to the inhibition of  $\alpha$ -synuclein transmission. In an  $\alpha$ -synuclein-enriched cellular model, MSCs inhibited the internalization of extracellular  $\alpha$ -synuclein and decreased the transmission of  $\alpha$ -synuclein in a donor-acceptor model. As a result, MSCs significantly decreased the levels of internalized cytosolic  $\alpha$ -synuclein and led to attenuation in  $\alpha$ -synuclein-induced cell death. Specifically, we demonstrated that MSCs can inhibit the CME of extracellular  $\alpha$ -synuclein fibrils via modulation of interaction between  $\alpha$ -synuclein and NMDA receptors.  $\alpha$ -Synuclein treatment led to increased expression of clathrin and EEA1, with a concomitant decrease in the immunoreactivity of surface NR1 and NR2A subunits. Meanwhile, MSC treatment significantly attenuated the  $\alpha$ -synuclein-induced expression of clathrin and EEA1, as well as an interaction between  $\alpha$ -synuclein and NMDA receptors. In an animal model of  $\alpha$ -synuclein inoculation, MSC treatment markedly decreased the transmission of extracellular  $\alpha$ -synuclein regions of the brain at a distance from the inoculation site. When  $\alpha$ -synuclein was inoculated in the cortex, MSC treatment markedly decreased the density of internalized  $\alpha$ -synuclein and the pathogenic phosphorylated form of  $\alpha$ -synuclein in both the ipsilateral and contralateral hemispheres. Moreover, MSC administration in  $\alpha$ -synuclein-inoculated mice significantly decreased the expression of clathrin, which was followed by decreased expression of EEA1 and an interaction between  $\alpha$ -synuclein and NMDA receptors of NR1 and NR2A. Additionally, when  $\alpha$ -synuclein was inoculated in the striatum, MSC treatment decreased the phosphorylated form of  $\alpha$ -synuclein expression in the midbrain at 30 days after inoculation. Consequently, modulation of extracellular  $\alpha$ -synuclein propagation by MSCs led to the exertion of a prosurvival effect on cortical neurons and nigral dopaminergic

### Figure 7. Long-Term Effects of MSCs on Extracellular $\alpha$ -Synuclein Transmission

(A) The immunoreactivity of phosphorylated form of  $\alpha$ -synuclein (p- $\alpha$ S) in the midbrain, showing that p- $\alpha$ S immunoreactivity was markedly attenuated MSC-treated animals compared to  $\alpha$ -synuclein ( $\alpha$ S)-inoculated animals. Scale bar represents 10  $\mu$ m. Arrowheads denote co-localization of Alexa Fluor 488-labeled  $\alpha$ S with p- $\alpha$ S.

(B) The immunoreactivity of p- $\alpha$ S in the TH-positive neurons of the SN was markedly attenuated MSC-treated animals compared to  $\alpha$ S-inoculated animals. Scale bar represents 10  $\mu$ m. Arrowheads denote co-localization of TH with p- $\alpha$ S.

(C) The number of TH-positive neurons in the SN at 30 days (d) after  $\alpha$ S inoculation. L, left; R, right. Scale bar represents 10  $\mu$ m.

(D) Behavioral analysis, showing that  $\alpha$ S inoculation led to progressive patterns of the latency to fall on the Rotarod test and increased latency to descend at 30 days after  $\alpha$ S inoculation on fall test compared to the control group, whereas MSC treatment restored impaired motor coordination and balance on the Rotarod test and pole test (n = 5 per group).

(E) The distribution of p- $\alpha$ S accumulations in the striatum, parietal cortex, and entorhinal cortex, showing that MSC treatment in  $\alpha$ S-inoculated animals significantly attenuated the immunoreactivity of p- $\alpha$ S in these areas. Scale bar represents 10  $\mu$ m.

All data are presented as means  $\pm$  SE. \*p < 0.05; \*\*p < 0.01.



neurons, with functional improvement of impaired motor coordination and balance against an  $\alpha$ -synuclein-enriched environment.

Interestingly, we demonstrated in the present study that the MSC-derived factor, Gal-1, could inhibit transmission of  $\alpha$ -synuclein through competition with  $\alpha$ -synuclein for NMDA receptor binding, leading to reduced endocytosis and thus lower  $\alpha$ -synuclein entry. Gal-1, a galactose-binding lectin, is a multifunctional molecule involved in the regulation of cell adhesion, cell proliferation, and programmed cell death (Perillo et al., 1998). In the nervous system, Gal-1 is involved in the proliferation of neural stem cells, neritic outgrowth, and cellular adaptation of redox status (Lekishvili et al., 2006; Miura et al., 2004), as well as the regulation of glutamate toxicity via interaction with the NR1 subunit (Lekishvili et al., 2006). In an  $\alpha$ -synuclein-enriched cellular model, Gal-1 treatment significantly decreased the expression of clathrin and EEA1 and increased the expression of NR1, with a concomitant increase in NMDA receptor binding, which led to decreased levels of internalized cytosolic  $\alpha$ -synuclein. This modulatory effect of Gal-1 on transmission of aggregated  $\alpha$ -synuclein was further supported by Gal-1 siRNA treatment in vitro, showing that siRNA counteracted the inhibitory effect of MSCs on CME of  $\alpha$ -synuclein via NMDA receptors and its associated prosurvival effects on SH-SY5Y cells. In vivo data showed that Gal-1 was co-expressed within MSCs injected intravenously and that Gal-1 treatment blocked CME of extracellular  $\alpha$ -synuclein fibrils by inhibiting interaction of  $\alpha$ -synuclein and surface NMDA receptors. In animals treated with enriched  $\alpha$ -synuclein, this modulatory effect of Gal-1 on membrane trafficking of  $\alpha$ -synuclein seemed to be comparable to those of MSCs by showing that Gal-1 treatment markedly decreased the extent of inoculated  $\alpha$ -synuclein aggregates, as well as expression of the pathological  $\alpha$ -synuclein, compared to fresh medium. In this regard, the present study provides evidence that Gal-1, as an MSC-derived soluble factor, can modulate the pathogenic microenvironments of extracellular  $\alpha$ -synuclein via modulation of CME.

Although the exact mechanism contributing cell-to-cell transmission of  $\alpha$ -synuclein is unknown, several possible routes mediated by direct penetration, fluid-phase, or receptor-mediated endocytosis, the form of exosome, or nanotube have been suggested depending on the forms of  $\alpha$ -synuclein. Of those, receptor-mediated endocytosis, which requires specific interactions between ligands and cell-surface receptors, seems to be a major mode of fibril internalization (Guo and Lee, 2014). Along with evidence of  $\alpha$ -synuclein participation in CME (Cheng et al., 2011), several studies suggested that forms of  $\alpha$ -synuclein with a higher molecular weight, such as aggregated fibrillar or oligomeric forms, may be internalized through an endocytic pathway via receptor (Chai et al., 2013; Cheng et al., 2011; Lee et al., 2008, 2010b, 2014). Moreover, other studies have shown that the receptor-mediated endocytosis is also involved in amyloid- $\beta$  transmission. Amyloid- $\beta$  could decrease the surface expression of NMDA receptors by promoting endocytosis of receptor proteins (Sakono and Zako, 2010; Snyder et al., 2005), which is quite in accordance with the present result. Additionally, the laminin receptor has a central role in mediating the internalization of amyloid- $\beta$  (Da Costa Dias et al., 2014). Taken together, the present data indicate that MSCs have a prosurvival effect

on cortical and dopaminergic neurons against propagation of  $\alpha$ -synuclein by modulating a major route of transmission of toxic protein aggregates. However, the data in the present study do not directly support the modulatory effect of MSCs on cell-to-cell transmission, as our in vitro experiment does not fully address aggregated  $\alpha$ -synuclein in cellular systems.

According to our in vitro data, the modulatory effect of MSCs on extracellular  $\alpha$ -synuclein and their prosurvival effects on SH-SY5Y cells were not completely blocked by Gal-1 siRNA-treated CM. Additionally, even though MSCs significantly interacted with both NR1 and NR2A subunits, Gal-1 treatment did not show a significant interaction with the surface NR2A subunit. These results suggest the existence of other MSC-derived soluble factors involved in aggregated  $\alpha$ -synuclein transmission. Therefore, further studies are required to identify the MSC-derived small molecules or exosomes responsible for extracellular  $\alpha$ -synuclein modulation that would have clinical potential for the development of disease-modifying therapeutic strategies for  $\alpha$ -synucleinopathies.

In summary, the present data indicated that MSCs exert neuroprotective properties through inhibition of extracellular  $\alpha$ -synuclein transmission. In addition, Gal-1 may be the principal soluble factor released by MSCs responsible for modulation of extracellular  $\alpha$ -synuclein. With advantages in clinical applications (Bang et al., 2003; Lee et al., 2012a), the use of MSCs or MSC-derived soluble factors as pharmacological modulators of  $\alpha$ -synuclein propagation may be an effective therapeutic approach.

## EXPERIMENTAL PROCEDURES

### MSCs and SH-SY5Y Culture

Frozen vials of characterized human MSCs at passage 2 were obtained from the Severance Hospital Cell Therapy Center. The human neuroblastoma cell line, SH-SY5Y, was obtained from the Korean Cell Line Bank. Both MSCs and SH-SY5Y cells were maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum (HyClone) and an antibiotic mixture of penicillin and streptomycin (1%, HyClone). When these cells reached 70%–80% confluence, they were trypsinized and subcultured. These cells were cultivated in a humidified incubator at 37°C and 5% CO<sub>2</sub> before use. For differentiation, SH-SY5Y cells were plated at a density (of  $5 \times 10^5/\text{cm}^2$ ) and grown as a monolayer in DMEM. One day after plating, the cells were incubated in fresh DMEM with 10  $\mu\text{M}$  retinoic acid (Sigma). The medium was changed on alternate days, and cultures were allowed to differentiate for 2 weeks (Lopes et al., 2010) and were then treated with  $\alpha$ -synuclein (1  $\mu\text{M}$ ) or Gal-1 (100 ng) for 2 hr. Additionally, the effects of MSCs were tested in differentiated SH-SY5Y that were co-cultured without direct contact using a Costar transwell (Corning). The MSCs were cultured on the permeable membrane of a Costar transwell insert, and the differentiated SH-SY5Y cells were maintained on the bottom of a plate. For inhibition of  $\alpha$ -synuclein clearance, bafilomycin A1 (50 nM, Sigma) was added to the medium containing  $\alpha$ -synuclein. For inhibition of endocytosis and NMDA receptors, dynasore (80  $\mu\text{M}$ , Sigma) and MK-801 hydrogen maleate (50  $\mu\text{M}$ , Sigma) were pre-treated to the replacement medium for 3 hr. Finally, Pitstop 2 (30  $\mu\text{M}$ , Abcam) was pre-treated to the replacement medium for 10 min to inhibit CME, and as a selective NR1/NR2A receptor inhibitor (Hansen et al., 2012), TCN 201 (50  $\mu\text{M}$ , Tocris) was pre-treated to the replacement medium for 3 hr. All experiments were replicated three times.

### BiFC System

SH-SY5Y cells were transfected with Venus1- $\alpha$ -synuclein or  $\alpha$ -synuclein-Venus2 (Bae et al., 2014) using electroporation. The stable cell lines were maintained with 200  $\mu\text{g}/\text{ml}$  G418 (Invitrogen).

### Preparation of Cell CM

CM were prepared as follows: 80% confluent MSCs at passage 5 and SH-SY5Y cells were fed with serum-free DMEM for 72 hr. The media of MSCs and SH-SY5Y cells were both assumed to contain various paracrine molecules. These media were collected. For *in vivo* injection, CM from  $1 \times 10^6$  cells were centrifuged to remove cell debris and concentrated using VIVASPIN6 centrifugal filter devices (Sartorius Stedim) at 4°C for 1 hr. These media were stored frozen.

### Cell Viability Analysis

Differentiated SH-SY5Y cells were harvested and plated in 24-well polystyrene plates (Corning) at  $1.5 \times 10^5$  cells. Plates were incubated at 37°C for 24 hr to allow cells to attach. After 24 hr, SH-SY5Y cells were directly treated with  $\alpha$ -synuclein. The same volume of DMEM was added to the control cultures. Plates were then incubated at 37°C for an additional 3 hr, 24 hr, and 48 hr. Cell viability was measured by trypan blue exclusion assays. Briefly, after the cells were incubated with the various medium samples, SH-SY5Y cells were washed with PBS and trypsinized, and then 10  $\mu$ l of cells were added to the trypan blue and mixed thoroughly. The viable cells were counted using a Countess automated cell counter (Invitrogen). The mean value of Ki67-negative cells in randomly five chosen fields was calculated by respectively counting using the 40 $\times$  magnification objective lens. Values of cell viability were expressed as calculated number of Ki67-negative cells counted/total cells counted  $\times 100$  = a percentage of viability. All experiments were repeated at least three times.

### Animal Study

All procedures were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experimentation provided by the Institutional Animal Care and Use Committee (IACUC) at the Yonsei University Health System. Male C57BL/6 mice (Orient Bio) were acclimated in a climate-controlled room with a constant 12 hr/12 hr light/dark cycle for 1 week prior to the initiation of drug administration. At 6 weeks of age,  $\alpha$ -synuclein (5  $\mu$ g per mouse) was administered via the neocortex. Cortical administration was carried out in accordance with the procedure described previously, with minor modifications (Hansen et al., 2011). Briefly, mice were anesthetized with isoflurane (Baxter), and  $\alpha$ -synuclein was slowly injected bilaterally into the cortex (0.4 mm posterior to bregma,  $-1.3$  mm lateral to midline, and  $-0.6$  mm ventral to the brain surface) using a stainless steel (26-gauge) injection needle connected to a 10- $\mu$ l microsyringe (Hamilton). The needle was left in place for 10 min before being withdrawn slowly. To evaluate the short-term effects of MSCs on  $\alpha$ -synuclein transmission, the mice were randomly divided into three groups ( $n = 5$  per group): (1) control; (2)  $\alpha$ -synuclein; and (3)  $\alpha$ -synuclein and MSC. Control mice were injected with saline via tail vein at 1 day after  $\alpha$ -synuclein inoculation (postoperative day 1). Mice in the MSC group were subjected to MSCs into the tail vein ( $1 \times 10^6$  cells per 200  $\mu$ l) on postoperative day 1. All mice were sacrificed on postoperative day 7. Additionally, to evaluate the modulation of  $\alpha$ -synuclein transmission by MSC-CM or Gal-1, the mice were randomly divided into six groups ( $n = 5$  per group): (1) fresh medium; (2) MSC-CM; (3) Gal-1; (4) Gal-1 siRNA-CM; and (5) CD105 siRNA-CM. Mice in the each group were subjected to medium delivery on postoperative day 1, and all mice were sacrificed on postoperative day 7. Finally, to evaluate the long-term effects of MSCs on  $\alpha$ -synuclein transmission, the mice were randomly divided into three groups ( $n = 5$  per group): (1) control; (2)  $\alpha$ -synuclein; and (3)  $\alpha$ -synuclein and MSC.  $\alpha$ -Synuclein was slowly injected into the striatum (0.2 mm posterior to bregma,  $\pm 2.0$  mm lateral to midline, and  $-2.6$  mm ventral to the brain surface). Mice in the MSC group were subjected to MSCs into the tail vein ( $1 \times 10^6$  cells per 200  $\mu$ l) on postoperative day 1 and postoperative day 10. All mice were sacrificed on postoperative day 30.

### Depletion of M1 or M2 Microglia

Microglia depletion was carried out in accordance with the procedure described previously, with a minor modification (Lee et al., 2012b; Miron et al., 2013). Gadolinium chloride (Sigma) and mannosylated-clodronate-encapsulated liposomes (Encapsula Nano Sciences) were applied to produce the M1 and M2 microglia depletion conditions, respectively. Gadolinium chlo-

ride (270  $\mu$ M) was stereotactically injected into the cerebral ventricle (0.2 mm posterior to bregma, 1.0 mm lateral to midline, and 3.0 mm ventral to the brain surface) of mice, followed by stereotaxic injection of clodronate liposomes (270  $\mu$ M) into the cerebral ventricle. Next,  $\alpha$ -synuclein was slowly injected into the cortex on postoperative day 5.

### Immunoprecipitation Assay

Membrane or cytosol lysates were obtained as described in the [Supplemental Experimental Procedures](#). NR1, NR2A, and Gal-1 antibodies were added to 1 mg of lysates and incubated for 4 hr at 4°C. Protein G agarose beads (Invitrogen) were added and incubated for overnight at 4°C. Then, the lysates were spun at 14,000  $\times g$ , the pelleted protein G agarose was washed by lysis buffer five times, and the pellet was resuspended with 20  $\mu$ l 2 $\times$  SDS sample buffer and subjected to SDS-PAGE and western blot analysis with  $\alpha$ -synuclein antibody.

### Measurement of $\alpha$ -Synuclein

The amount of  $\alpha$ -synuclein was measured using a sandwich ELISA kit (AnaSpec). 10  $\mu$ l of each diluted sample, and standards included in the kit were applied to microtiter plates precoated with antibody that specifically recognized  $\alpha$ -synuclein. Following an overnight incubation at 4°C and washing, a detection antibody indirectly linked to an enzyme was applied. After incubation and washing, 350  $\mu$ l of wash solutions were added to each well and then the plate was inverted and dried by hitting the plate until no moisture appears. The substrate was added and incubated for 15 min at 37°C, and then the reaction was stopped with stop solution. The color reaction was measured with an automatic ELISA microplate reader (BioTek Instruments), with the wavelength set at 450 nm. The software (Bio-Rad) was used to create standard curves and to calculate the concentration of the samples.

### Cytokine Analysis in MSC-CM

MSC-CM and Gal-1 siRNA-treated MSC-CM were assayed in duplicate using specific ELISA kits for human IL-4, IL-6, IL-10, HGF, CCL2, TGF- $\beta$ 1, VEGF, and IFN- $\gamma$ , respectively (R&D Systems). The optical density of each well was read at 450 nm and corrected at a wavelength of 540 nm according to the manufacturer's instructions with an automatic ELISA microplate reader (BioTek Instruments). The final concentration was calculated by converting the optical density readings against a standard curve.

### Additional Procedures

Please refer to the [Supplemental Experimental Procedures](#) for details on  $\alpha$ -synuclein aggregate fluorescent dye labeling, plasmid transfections, 2D-PAGE, MALDI-TOF/MS analysis, RT-PCR, brain sample preparation, immunocytochemistry, immunohistochemistry, western blotting analysis, rotarod test, pole test, TUNEL assay, and stereological cell counts.

### Statistical Analysis

The group means were compared using the Mann-Whitney U test for pairs and the Kruskal-Wallis analysis for multiple groups. *p* values less than 0.05 were considered statistically significant. Statistical analyses were performed using commercially available software (version 12.0; SPSS).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.12.075>.

### AUTHOR CONTRIBUTIONS

S.H.O. and H.N.K. conceived the study, performed all experiments, and wrote the manuscript. H.J.P., M.K.S., and J.Y.S. provided technical help. E.-J.B. provided  $\alpha$ -synuclein. S.-J.L. discussed results and commented on the manuscript. P.H.L. supervised the entire study, interpreted data analysis, provided financial support, and gave final approval of the manuscript.

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